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FR 002654113 A1
J.Virol.Methods 1993,41(3),311-322 J.Med.Virol. 1991, 33(4),260-267 Frontiers of Virology - Diagnosis of Human Viruses by PCR, Springer-Verlag(1992),pages 355 to 371

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(54) Oligonucleotide sequences for the detection of flaviviral RNA

(57) A method for determining the presence of flaviviral RNA comprises carrying out a reverse transcription using a sample suspected of containing denatured viral RNA as template source and a transcription primer having a sequence selected from any consecutive 10 bases or more of the nucleotide sequence (I):

5'-TGTATGCTGATGACACAGCAGCAGGATGGGACAC-3' (I)

determining the sequence of all or part of the transcription product, and relating the presence of sequences which hybridise to an oligonucleotide having a nucleotide sequence selected from any consecutive 10 bases or more of the nucleotide sequence II:

5'-TCAAGGAACTCCACACATGAGATGTACT-3' (II)

to the presence of a flaviviral RNA.

There is also described oligonucleotides comprising at least 10 consecutive bases selected from (I) or (II) or sequences thereof having equivalent hybridisation specificities.

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VIRAL DIAGNOSTIC TEST

The present invention relates to a method for identifying viral RNA, or RNA or DNA derived therefrom by reverse transcription, to specific amplification primers for use therein, their use as probes, and to test kits comprising such probes and/or primers. Specifically the present invention provides such method, primers, probes and kits for the generic identification of flaviviruses, whereby the presence of such viral RNA, DNA transcribed from it, and ultimately the viruses or viral infection, can be detectected in a single test.

The flaviviruses are single stranded RNA viruses. Many are medically important in man, including dengue (Den) fever, yellow fever (YF), Japanese encephalitis (JE) and tick-borne encephalitis (TBE), while several of medical importance are rarer, such as Rocio which causes a disease with similar clinical symptoms to St. Louis encephalitis and JE. Many other flaviviruses cause diseases in humans which are not of major public health importance but must be considered in the diagnosis of febrile illness in persons inhabiting or travelling to endemic areas eg Banzi, Ileus while many others are not well characterised due to the lack of research and medical facilities in the geographical areas affected. Flaviviruses also cause disease in animals of economic importance for example, louping ill (LI) and Wesselsbron (WSL) and occasionally these can be transferred to man.

Initial diagnosis of flavivirus infections is often based on the medical history of the infection and the clinical symptoms, but this approach may not be reliable due to their range and complexity. Specific diagnosis of the most common flavivirus infections can be by virus isolation or serological testing, but often virus isolation is only possible from brain tissue. The isolated viruses may be grown in appropriate cell lines, which can take at least 3 days, and are then identified by immunofluoresence. However, most common flavivirus infections are specifically diagnosed using serological tests.

In some cases the serum sample must be taken at an appropriate time for the test to be reliable. Serological tests used include haemagglutination inhibition, complement fixation, neutralization and IgM capture ELISA. The interpretation of such serological tests can prove difficult and it is known that many flavivirus antibodies cross-react and the prior use of flavivirus vaccines or previous infections may also confuse serodiagnosis. Thus there is a clear requirement for a rapid, reliable, simple and inexpensive diagnostic method for flaviviruses.

The polymerase chain reaction has been shown to be useful for the detection of some of the common strains eg. Den (Henchal et al.,1991; Lanciotti et al.,1992; Lewis et al.,1992), SLE, JE and YF (Eldadah et al.,1991; Howe et al.,1992), but has not been developed for most. Pan-flavivirus detection and diagnosis could be used, (i) to confirm the diagnosis from the clinical symptoms wherein, if required this initial rapid diagnosis could then be followed by more specific diagnosis where available; (ii) to aid in the diagnosis of the rarer flavivirus infections for which diagnosis is still non-routine; and (iii) some nonspecific viral infections could then be identified as flavivirus infections.

The use of such a pan-flavivirus PCR has been reported by Trent and Chang in 'Frontiers of Virology-Diagnosis of Human Viruses by PCR (1992) Edited by Y Becker and G Barai- Springer Verlag: see pages 355-371'. These workers report that by carrying out a Reverse transcription/polymerase chain reaction targeted at an 832-base pair region of the NS5 gene they can readily generically identify 42 known flaviviridae using two highly conserved sequences as targets for PCR primers once transcription of native RNA has been carried out. The target sequences are stated to have maximum homology for all viruses tested, low homology with other genes in databases, high melting temperature, minimum primer dimer interaction and to provide amplification of DNA with of relatively short fragment length

while still containing variable regions allowing specific virus type identification. Amplimer probes were designed to have minimum sequence homology with other flaviviruses, high melting temperature, minimum homodimer and heterodimer interaction, and virus specific sequence regions located between 9166 and 9977.

The present inventors have now identified a further set of specific sequence amplification targets, also derived from the NS5 gene, that allow generic identification of the flaviridae, which may be used with the prior art generic method to provide enhanced certainty of identification of these viruses with increased confidence where previously unidentified or uncharacterised organisms are encountered. These targets may also be used on their own to provide such identification as an alternative to the prior art.

Thus, in a first aspect thereof, the present invention provides a method for determining the presence of flaviviral RNA comprising carrying out a reverse transcription using a sample suspected of containing denatured viral RNA as template source and a transcription primer having a sequence selected from any consecutive 10 bases or more of the nucleotide sequence (I):

5'-TGTATGCTGATGACACAGCAGGATGGGACAC-3' (I)

determining the sequence of all or part of the transcription product, and relating the presence of sequences which hybridise to an oligonucleotide having a nucleotide sequence selected from any consecutive 10 bases or more of the nucleotide sequence II:

5'-TCAAGGAACTCCACACATGAGATGTACT-3' (II)

to the presence of a flaviviral RNA.

Particularly provided is a preferred method of the invention wherein

the determination of part of the sequence is carried out by carrying out a PCR reaction using the double stranded transcription product as template and using primers comprising any 10 or more consecutive bases of the sequences (I) and (II) respectively, whereby presence of sequences hybridising to the sequence (II) is confirmed by observation of PCR products of about 1000 (eg. 800-1200) basepairs in size. Use of the 10 base length primers of the sequences (I) and (II) nearest the 5' end or the entire sequences will result in about a 1018 basepair product for a positive result with yellow fever, whereas use of the 10 nearest the 3' end will result in a 981 basepair product.

The products provided by the reverse transcription and the preferred RT/PCR of the present invention are convenient substrates for steps which might identify the specific flavivirus in a further step, eg. employing nested primer amplification, nucleotide probing, LCR, restriction mapping etc. Precise size will be isolate specific.

It will be realisedby those skilled in the art that for use as both transcription or PCR primers in the aforesaid methods of the invention, the sequences above allow that the bases TCAG have their usual meaning, but the invention clearly also provides equivalent sequences wherein one or more bases have been substituted with analogues having equivalent hybridization specificity.

It will be realised that the 5' ends of the primers derived from sequences (I) and (II) may be extended to include various sequences which allow manipulations and or interactions as required, but that the 3' sequences should not comprise bases that would inhibit their hybridization with their respective target NS5 and complementary sequences. Preferably the primers will consist of only ten or more consecutive bases selected from the specified sequences, and most preferably consist of the sequences (I) and (II) themselves.

In a second aspect of the invention provides oligonucleotides

comprising 10 or more consecutive bases from sequences (I) or (II), with optional extensions in the 5' and/or 3' direction on condition that such extension in the 3' direction do not inhibit hybridization to target NS5 sequence and its complementary sequence respectively.

The reverse transcription step of the present invention is conveniently carried out after denaturing any virus and RNA present in the sample by treating it to a denaturising treatment, eg. by use of heat. Reverse transcription is conveniently carried out by standard methods while specific sequence amplification is carried out most conveniently using polymerase chain reaction.

The methods and primers of the present invention will now be described by way of illustration only by reference to the following non-limiting examples; further embodiments will occur to those skilled in the art on the light of these.

Materials and Methods

Viruses

Virus strains used in this study were obtained from the NERC, Institute of Virology and Environmental Microbiology, Oxford, UK, Centre for Applied Microbiology Research (CAMR), Salisbury,UK and the University of Surrey, Guildford, UK. (See Table 1 which indicates the publications in which disclose the virus types referred to).

Propagation of virus and extraction of vRNA

Viruses were propagated in Vero cells at a multiplicity of infection of approx. 0.1. When an obvious cytopathic effect was seen, the cell monolayers were washed twice in cold phosphate buffered saline (0.1M phosphate, 0.9% NaCl pH 7.2; PBS), resuspended in cold PBS and subjected to one further wash before resuspension in 900µl disruption buffer (0.15M NaCl, 0.1M tris pH 7.5, 1.0M EDTA).

Placental RNAse inhibitor (25U/ml, Sigma Chemical Company Ltd., UK) was added, and after swelling for 10 min at 37°C the pellet was digested by the addition of NP40 to a final concentration of 1%, and vortexing. After removal of the nuclei by centrifugation, total RNA was extracted from the supernatant by the phenol/chloroform method, and precipitated with ethanol at -20°C. During the first phenol /chloroform extraction the samples were heated to 60°C for 15 min.

Primer synthesis

The oligonucleotides were synthesised using an Applied Biosystems DNA synthesizer (ABI 392) and resuspended in HPLC grade water (Aldrich Chemical Company, England) before use.

EXAMPLE 1

Reverse Transcription and Polymerase Chain Reaction (RT/PCR)

Each viral RNA (vRNA) sample was denatured by boiling for 5 min and cooling on wet ice for 5 min. 500ng of primer FG2 was mixed with the denatured vRNA and the mix was boiled for a further 5 min and cooled on wet ice. The RT reactions were performed in 9µl vols containing the vRNA/primer FG2 mix and 5mM TrisHCl pH 8.3, 5mM KCl, 10mM $MgCl_2$, 3mM dithiothreitol, 0.1% Nonidet P40, 0.7U/µl RNAsin (Boehringer Mannheim UK), 1mM each of the four deoxynucleotide triphosphates and 0.75U/µl reverse transcriptase RAV2 (Amersham International, Buckinghamshire, England). The reactions were incubated at 42°C for 2 h or 56°C for 1 h (Lewis et a1.,1992).

Half the RT reaction mix was then used as template in the subsequent PCR. 500ng primer FG1 and 500ng primer FG2 were added to the template. This mixture was boiled for 5 min cooled on wet ice then heated to 94°C in a thermocycler (Perkin-Elmer Cetus) before the addition of 5µl x10 Taq buffer (Boehringer Mannheim, UK) and 0.6U Taq polymerase (BoehringerMannheim, UK). The final volume of the reaction mix was adjusted to 50 µl with HPLC pure H_2O (Aldrich Chemicals Ltd).

The reaction mixes were incubated at 94°C for 4 min, 40°C for 2 min, 72°C for 3 min, followed by five cycles of 95°C for 1 min, 40°C for 2 min, 72°C for 3 min. Then thirty cycles of 95°C for 1 min, 45°C for 3 min, 72°C for 3 min preceded by a final cycle of 95°C for 1.5 min, 45°C for 2 min, 72°C for 10 min. For some reactions the mixes were cycled at 94°C for 4 min, then twenty five cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 10 min and a final incubation of 72°C for 20 min. After cycling, reaction products were visualised after agarose gel electrophoresis and ethidium bromide staining (Sambrook et al.,1989).

Cloning of RT/PCR products

DNA bands were excised from the agarose gel using a Geneclean kit (Strategene) according to the manufacturers instructions and ligated with pUC18 which had been digested with SmaI and treated with bacterial alkaline phosphatase (Boehringer Mannheim, UK) (Sambrook et al.,1989). The mix was transformed into E.coli Sure cells (Strategene) and plated onto L-agar containing ampicillin, IPTG and X-GAL (Sambrook et al.1989). After incubation for 18 hr at 37°C colonies containing recombinant plasmids were identified by PCR of the insert using the conditions described above with the reverse and -40 M13 primers. Plasmid DNA was extracted from bacterial cells using the alkalinelysis method (Sambrook et al.,1989).

Sequencing

Single stranded DNA was produced using Dynabeads(RTM) (Dynal A.S., Oslo, Norway) using a method described by the manufacturer. Nucleotide sequencing was carried out with a taq dyedeoxy(RTM) terminator cycle sequencing kit following the manufacturers instructions (applied Biosystems). Samples were electrophoresed through a 6% acrylamide gel (373A DNA Sequencer, Applied Biosystems). Nucleotide sequence data was analyzed using DNASTAR software.
All of the flavivirus genomes for which the nucleotide sequence data is available appear to have a similar structure to the YF virus genome

which is composed of three structural genes and seven non-structural genus (Rice et al: (1985) Nucleotide sequence of yellow fever virus implications for flavivirus gene expression and evolution, Science, 229: 726-733). Other workers have gone some way in the past to determine conserved regions of some flaviviruses (eg. Pletnev et al. (1990) Nucleotide sequence of the genome and complete amino acid sequence of the polyprotein of tick-borne encephalitis virus, Virology, 174: 250-263. Mandl et al (1989) Genome sequence of tick-borne encephalitis virus western subtype and comparative analysis of non structural proteins with other flaviviruses, Virology, 173: 291-301).

The present primers have been selected such that they are at least 75% identical with each sequence in the alignment in Table 1. The maximum identity was 98% and for some nucleotide positions in the primer the consensus nucleotide was not chosen, to ensure that the overall identity of the oligonucleotide with all NS5 sequences was maximised. In each case the oligonucleotides could pair with every sequence in the alignment at the 3'-base.

Screening the EMBL/Genebank databases for homology with these oligonucleotides revealed some nucleotide sequences which contained one or both of the conserved regions. However, none of these nucleotide sequences were predicted to cross-react with both primers in a similar manner to the flavivirus NS5 genes.

EXAMPLE 2: Amplification of Kumlinge Virus NS5 Gene Fragment

Primers (I) and (II) were used in a RT/PCR to amplify a fragment from Central European-tick-borne encephalitis virus Kumlinge (Kum) vRNA. A single band of the expected size (approx. 1Kb) was generated. The amplified DNA was subjected to a further round of PCR and the product of this reaction was cloned into a plasmid vector. Four recombinant plasmids were sequenced in both directions to generate a consensus sequences for 100 base pairs at the 3' and 5' ends of the insert.

Comparison of these consensus sequences with the sequence of the CE-TBE Neudoerfl NS5 gene (Mandl et al.1989) indicated that the ends of the cloned PCR product had 96% homology with nucleotides 8328 to 8427 and 100% homology with 9155 to 9244. Thus we concluded that the RT/PCR fragment spanned the correct region of the NS5 gene of CE-TBE Kum virus.

EXAMPLE 3-16: Amplification of Other Flavivirus vRNAs

Oligonucleotides (I) and (II) were used in a RT/PCR with other flavivirus vRNAs and appropriate negative controls (Table 2). The test panel was chosen to be representative of flavivirus vRNAs isolated from viruses across the genus including viruses transmitted by ticks, mosquitos and non-arthropod vectors. Each of the test panel vRNAs were detected by a single DNA fragment of the expected size. Further more this result was also obtained from YF vRNA isolated from tissue culture fluid and tissue samples. No amplified DNA fragments were observed from any of the other nucleic acids tested in the reaction.

These results were obtained from a selection representing only approx 20% of all flaviviruses. However, the viruses in the test panel can be considered representative of this group in terms of the diseases they cause, their mode of infection and their genetic relatedness Blok et al studied the relatedness of all flavivirus genes based on published sequence information and the panel includes representatives of the least related viruses, Den and TBE and various viruses between such as YF, Kunjin and Murray Valley encephalitis. It also included some flaviviruses for which little or no sequence information is available, notably the non-vector borne flaviviruses Modoc and Rio Bravo. Thus, it is likely that the conserved sequences are present in the genomes of many flaviviruses and perhaps all of them.

TABLE 1 (a) Comparison of primers (I and II) and flaviviral sequences

PRIMER I	5' T GTA TGC TGA TGA CAC AGC AGG ATG GGA CAC 3'
SOURCE	- = matched
Den-1a	- --- --- A-- --- --- --- C-- --- --- T--
Den-2b	- --- --- C-- --- --- C-- --- --- --- ---
Den-2c	- --- --- C-- --- --- C-- --- C-- --- ---
Den-2d	- --- --- C-- --- --- C-- --- --- --- T--
Den-3e	- --- --- --- --- --- --- C-- T-- --- ---
Den-4f	- --- --- --- --- --- --- --- C-- --- ---
JEg	- --- C-- --- --- T-- C-- C-- G-- --- ---
KUNh	- C-- C-- --- --- --- --- C-- T-- --- ---
WNi	- T-- C-- --- --- T-- C-- --- C-- --- ---
YFj	- T-- --- A-- --- --- --- C-- C-- --- T--
CE-TBEk	- C-- --- A-- --- --- --- T-- C-- --- ---
FE-TBEl	- T-- --- A-- --- --- --- C-- C-- --- T--

PRIMER II	TCA AGG AAC TCC ACA CAT GAG ATG TAC T
SOURCE	- = matched
Den-1a	--- --A --T --T --T --- --A --- --T -
Den-2b	--- C-A --- --- --- --- --- --- --- -
Den-2c,d	--- C-- --T --- --- --- --- --- --- -
Den-3e	--- C-A --- --- --G --C --A --- --T -
Den-4f	--C --- --- --- --C --- --- --- --T -
JEg	--C C-A --- --- -AT --C --- --- --T -
KUNh	--- C-- --T --- --- --- --A --- --T -
WNi	--- C-C --- --T --C --- --- --- --- -
YFj	--C --- --T --- --T --- --A --- --- -
CE-TBEk	--G --- --- --A --C --- --- --- --T -
FE-TBEl	--- --- --- --- --C --- --- --- --T -

In the above table the anatations are indicative of the follwing sources:

a Fu et al (1992) Dengue Type 1 virus (Singapore strain S275/90), Virology, 188: 953-958.

b Deubel et al (1988) Dengue Type 2 virus, Virology, 165: 234-244. Irie et al (1989) (New Guinea-C strain), Gene, 75: 197-211.

c Hahn et al (1988) Dengue Type 2 virus, Virology, 162: 167-180.

d Blok et al (1992) Dengue Type 2 virus, Virology, 187: 573-590.

e Osatomi et al (1990) Dengue Type 3 virus, Virology, 176: 643-647

f Mackow et al (1987), Virology, 159: 217-228.

g Sumioshi (1987), Virology, 161: 497-510; Nitayaphan er al (1990), Virology, 177: 541-552; Aihara et al (1991) Virus Genes, 5: 95-109.

h Coia et al (1988) J. Gen. Virol., 69: 1-21.

i Castle et al (1986), Virology, 149: 10-26.

j Rice et al (see above), (1985).

k Mandl et al (1989), Virology, 173: 291-301.

l Pletnev et al (see above), (1990).

TABLE 2.

NUCLEIC ACID SOURCE	STRAIN	SOURCE	MODE OF TRANSMITION	DETECTED BY RT-PCR TEST
Banzi	SAH 336	IVEM	Mosquito	Yes
Dengue 2	TC992	IVEM	"	Yes
Dengue 2	New Guinea, C	SURREY	"	Yes
Ilheus	B52456	IVEM	"	Yes
Kunjin	3282	IVEM	"	Yes
Japanese encephalitis	Nakayama	IVEM	"	Yes
Murray valley encephalitis	MVE/1/1951	IVEM	"	Yes
Wesselsbron	Van Tonder	IVEM	"	Yes
Yellow fever	P16065	SURREY	"	Yes
Yellow fever	17D 204	CAMR	"	Yes
CE-TBE Kum virus	A59	CAMR	Tick	Yes
Powassan	L.B.	CAMR	"	Yes
Turkish Tick-borne encephalitis	not known	CAMR	"	Yes
Modoc	3321	IVEM	Non-arthropod	Yes
Rio Bravo	3360	IVEM	"	Yes
Vero cell total RNA	N/A	N/A	N/A	No
C6/36 total RNA	N/A	N/A	N/A	No
B.cereus DNA	NCTC 8075	N/A	N/A	No
E.coli DNA	JM101	N/A	N/A	No

CLAIMS.

1. A method for determining the presence of flaviviral RNA comprising carrying out a reverse transcription using a sample suspected of containing denatured viral RNA as template source and a transcription primer having a sequence selected from any consecutive 10 bases or more of the nucleotide sequence (I):

5'-TGTATGCTGATGACACAGCAGGATGGGACAC-3' (I)

determining the sequence of all or part of the transcription product, and relating the presence of sequences which hybridise to an oligonucleotide having a nucleotide sequence selected from any consecutive 10 bases or more of the nucleotide sequence II:

5'-TCAAGGAACTCCACACATGAGATGTACT-3' (II)

to the presence of a flaviviral RNA.

2. A method as claimed in claim 1 wherein the determination of the sequence of a part of the transcription product is carried out by performing a specific sequence amplification reaction using the double stranded transcription product as template and using primers comprising any 10 or more consecutive bases of the sequences (I) and (II) respectively, whereby presence of sequences hybridising to the sequence (II) is confirmed by observation of amplification products products of 800 to 1200 basepairs in size.

3. A method as claimed in claim 2 wherein the specific sequence amplification is a polymerase chain reaction.

4. A method as claimed in claim 1, 2 or 3 wherein the products provided by the reverse transcription and optional amplification are used as substrates for steps which might identify the specific flavivirus in a further step.

5. A method as claimed in claim 4 wherein the further step comprises employing nested primer amplification, nucleotide probing, LCR, or restriction mapping.

6. A method as claimed in any one of the preceding claims wherein the reverse transcription is carried out after denaturing any virus and RNA present in the sample by treating it by a denaturising treatment.

7. A method as claimed in claim 5 wherein the denaturising treatment comprises use of heat.

8. A method as claimed in any one of the preceding claims as described in Example 1, 2 or 3 herein.

9. An oligonucleotide hybridization probe, transcription primer or specific sequence amplification primer comprising a sequence selected from any consecutive 10 bases or more of the nucleotide sequence (I):

5'-TGTATGCTGATGACACAGCAGGATGGGACAC-3' (I)

or sequences of bases having equivalent hybridization specificities thereto.

10. An oligonucleotide hybridization probe or specific sequence amplification reaction primer comprising a nucleotide sequence selected from any consecutive 10 bases or more of the nucleotide sequence II:

5'-TCAAGGAACTCCACACATGAGATGTACT-3' (II)

11. An oligonucleotide as claimed in claim 9 or 10 having a nucleotide sequence consisting of a nucleotide sequence selected from any consecutive 10 bases or more of the sequences (I) or (II).

12. An oligonucleotide as claimed in claim 9 or 10 having a nucleotide sequence consisting of sequence (I) or (II).

13. A generic test kit for detection of flaviridae RNA comprising one or more oligonucleotides as claimed in any one of claims 9 to 12.

Patents Act 1977
Examiner's report to the Comptroller under Section 17 (The Search Report) 16

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Relevant Technical fields

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(ii) Int Cl (Edition 5) C12Q 1/68 1/70

Search Examiner
C SHERRINGTON

Databases (see over)

(i) UK Patent Office

(ii) ONLINE DATABASES: WPI, CLAIMS, CAS ONLINE, DIALOG/BIOTECH

Date of Search
4 OCTOBER 1993

Documents considered relevant following a search in respect of claims 1-13

Category (see over)	Identity of document and relevant passages	Relevant to claim(s)
A	FR 2654113 A1 (INSTITUT PASTEUR) - whole document	1-8
A	J Virol Methods 1993, 41(3), 311-322 Rapid identification of flavivirus using the polymerase chain reaction	1-8
A	J Med Virol 1991, 33(4), 260-267 Detection of flaviviruses by Reverse - transcriptase polymerase chain reaction	1-8
A	Frontiers of Virology - Diagnosis of Human Viruses by PCR, Y Becker & G Barai (1992, Springer-Verlag), pages 355-371 Detection and identification of flaviviruses by Reverse Transcriptase polymerase chain reaction	1-8

Category	Identity of document and relevant passages	Relevant to claim(s

Categories of documents

X: Document indicating lack of novelty or of inventive step.

Y: Document indicating lack of inventive step if combined with one or more other documents of the same category.

A: Document indicating technological background and/or state of the art.

P: Document published on or after the declared priority date but before the filing date of the present application.

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